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# CYTOCHEMISTRY OF THE LONG-NECKED CELLS IN THE FOOT OF *ONCHIDORIS MURICATA* (NUDIBRANCHIA)

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## Abstract

The long-necked cells of the foot on *Onchidoris muricata* were found distributed among the shorter secretory cells which composed most of the epidermal tissue. The long-necked cells extend through the basement membrane on which the smaller cells lie. The ultrastructure of the cell body at the base of the cell shows the nucleus, large amounts of rough endoplasmic reticulum, and an extensive Golgi body that produces large numbers of secretory granules. The periodic acid-thiosemicarbazide-silver protein (PA-TSC-SP) test for polysaccharides was negative for the secretory material. These results are consistent with polysaccharides having large obstructing groups such as sulfates. The high iron diamine (HID) test for sulfated carbohydrates was positive in the Golgi saccules and secretory granules at the base of the cell. Tannic acid fixation for glucosaminoglycans was only slightly positive in the extracellular matrix. Alcian blue stained tissue for light microscopy complimented the ultrastructural cytochemical tests and indicated that the secretory materials are sulfated mucopolysaccharides.

## Introduction

Although the ultrastructure of secretory cells producing various carbohydrate complexes has been thoroughly studied in mammalian systems, particularly in tissues specialized for fairly narrow functions, similar studies in gastropod secretory tissue are rare (Ovtracht, 1967; Ovtracht, *et al.*, 1969; Storch and Welsch, 1972; Chailley, 1979). Ultrastructural studies of multifunction secretory tissues in gastropods are even less common (Porter and Rivera, 1980).

The chemical components of mucoid materials secreted by various types of cells have been studied at the ultrastructural level by autoradiographic methods (Neutra and Leblond, 1966) and chemical stains (Thiéry, 1967; Friend, 1969; Spicer, *et al.*, 1978). Many cells containing secretory granules in epidermis of some nudibranchs are periodic acid Schiffs positive at the light optical level (Porter and Rivera, 1980). At the ultrastructural level, Porter and Rivera (1979) have demonstrated periodic acid oxidizable carbohydrates in the secretory granules of ceras epidermal cells of *Aeolidia papillosa.*

In this study, the large, secretory, long-necked cells of the foot of the nudibranch *Onchidoris muricata* were studied for their ultrastructural morphology and chemical constituents of the secretory granules. The limited distribution of these cells at the anterior of the foot generated an interest to determine the composition and function of long cell secretory product. The chemical analyses were primarily aimed at differentiating mucoid materials used for locomotion and adhesives for attachment to substrates (Cranfield, 1973a, 1973b).

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Abbreviations: PA-TSC-SP, periodic acid-thiosemicarbazide-silver proteinate; HID, high iron diamine; PAS, periodic acid-Schiffs.

## Materials and Methods

### *Nudibranch tissue*

*Onchidoris muricata* adults were collected from under rocks at low tide from King's Beach in Lynn, Massachusetts, and Rye Beach, New Hampshire, in the autumn of 1979. Whole animals were fixed with 5% glutaraldehyde in 0.075 *M* NaOH-PIPES (Piperazine-N,N′ bis [2-ethane sulfonic acid]) buffer pH 7.4 overnight at 4°C except for specimens used for extracellular matrix detection as noted below. The foot anterior was isolated and processed for light and electron microscopy.

### *Electron microscopy*

Small tissue blocks (2–3 mm on a side) of fixed foot anterior were stabilized at pH 6.8 in 0.075 *M* NaOH-PIPES buffer and then post stained in 2% osmium tetroxide in the same buffer for 1.5 h. The tissue was then dehydrated in an alcohol series and embedded in Spurr's plastic (Spurr, 1969). Ultrathin sections were obtained with a Porter-Blum Ultra-microtome MT-1. Observations and micrography were made with a Zeiss EM 9S-2 transmission electron microscope operated at 60 KV. All ultrathin sections were mounted on gold grids for all ultrastructural observations and electron microscopic cytochemical tests.

*Cytochemical stains*—Tests for periodate oxidizable carbohydrates were done as described by Thiéry (1967). In this procedure sections were oxidized for 30 min in periodic acid (PA). Thiosemicarbazone derivatization of oxidized carbohydrate was carried out with thiosemicarbazide (TSC) for 72 h. Silver was deposited at these sites after exposure of the sections to silver proteinate (SP) for 30 min. Appropriate controls leaving one or more reagents out of the reaction were employed to check the specificity of this test (Porter and Rivera, 1979).

For detection of sulfated carbohydrates, the high iron diamine (HID) procedure (Spicer *et al.*, 1978) was performed on small blocks (2 mm on a side) of fixed tissue. The tissue was stained in a freshly prepared solution of high iron diamine for 24 h at 20°C and then rinsed twice in 0.075 *M* NaOH-PIPES buffer pH 6.8, and post stained for 1 h with 2% $OsO_4$ in the same buffer. These tissues were dehydrated, embedded and sectioned as indicated above.

Extracellular matrix materials were observed by the addition of 2% tannic acid to a fixing solution for fresh tissue consisting of 2% paraformaldehyde and 1.5% glutaraldehyde buffered with 0.075 *M* NaOH-PIPES pH 5.2. Post fixation was done in 1% $OsO_4$ for 1 h (Singley and Solursh, 1980). Dehydration embedding and sectioning was carried out as with other specimens.

### *Light microscopy*

Fixed, isolated foot anterior cross sections were dehydrated in an ethyl alcohol series and then infiltrated with xylene. Xylene was substituted with paraffin and the tissues were cut into 10 $\mu$m thick sections and mounted on glass slides for cytochemical studies.

Alcian blue stains for determination of acid mucopolysaccharides were done by the methods of Pearse (1968). For detection of acidic mucosubstances, sections were exposed to freshly prepared 1% alcian blue 8 GX in 3% acetic acid for 30 min at pH 2.5. These sections were washed in running water, dehydrated in alcohol, cleared in xylene and mounted with Permount (Fisher Chem. Co., Fair Lawn, N. J.). Weakly acidic sulfated mucoids were stained with 1% alcian blue 8 GX in

0.1 *N* HCl for 30 min at pH 1.0. Sections were blotted with Whatman No. 50 filter paper and dehydrated, cleared, and mounted as above.

All light micrographs were taken on a Leitz Ortholux II interference contrast microscope equipped with a 35 mm camera.

## Results

### *Ultrastructural morphology*

The anterior sole of the foot in *Onchidoris muricata* consists of a ciliated secretory epithelium (Fig. 1). This epithelium is 20–30 μm thick and is a mixture of columnar ciliated cells and secretory gland cells. The narrow ciliated cells have a prominent centrally located nucleus and many mitochondria distributed in the cytoplasm toward the ciliated apex of the cell. A few vesicles are often present among the basal bodies of the cilia. In some of the ciliated cells large vacuolar spaces are present at the basal end near the basement membrane (Fig. 2). The secretory cells are usually wider than the ciliated cells and contain a greater variety of cytoplasmic organelles. Rough endoplasmic reticulum (ER), small vesicles and secretory granules are very common in the cytoplasm and easily detected. Golgi bodies are also present, but they are often out of the planes of section and, therefore, not visible in every cell. The basal vacuolar space found in ciliated cells is also present in the secretory cells (Fig. 1). In osmified tissues the product of the epidermal secretory granules is not easily distinguished from the product of subepidermal secretory cells. The neck or process of the subepidermal cells passing through the epidermis has contents similar to the mass of secretory granules of epidermal secretory cells (Figs. 1, 2). The two products are distinguished from one another with specific cytochemical stains (Figs. 8, 9). The epidermal cells rest on a basement membrane below which are various connective tissue cells surrounded by a considerable extracellular fibrillar component (Fig. 1).

The subepidermal long-necked secretory cells are found among the connective tissues below the basement membrane. All these cells are massed centrally in the foot and extend from one-third to two-thirds the distance from the anterior of the foot. The mass of long-necked cells is more widely distributed towards the anterior of the foot. The cell distribution then trails to a narrow point towards the foot posterior. The secretory cell bodies among the connective tissues are ovoids averaging 20 μm by 40 μm with the long axis perpendicular to the foot surface. A single narrow neck or process extends 45–70 μm from the cell body towards the surface of the foot. The neck penetrates the basement membrane and passes between the epithelial cells to the exterior of the foot (Fig. 1). The entire long-necked process contains tightly packed secretory granules that fuse as the secretory product is expelled from the apical end of the neck (Fig. 3).

The cell body also contains large numbers of secretory granules surrounding a centrally located nucleus, substantial amounts of rough ER, and a prominent Golgi body (Fig. 1, 4, 5). The secretory granule contents appear either fibrillar or granular (Fig. 5) in osmium post-fixed tissue. The Golgi body is usually found among the granules, commonly with progressively distending cisternae. The larger cisternae contained the fibrillar product which appear to change slightly into a granular material as the secretory granules mature and move distally from the Golgi body (Fig. 5). The proximal position of the expanding Golgi cisternae to the smaller secretory granules and the contents common to both organelles suggests secretory granule generation by the Golgi body (Fig. 5).

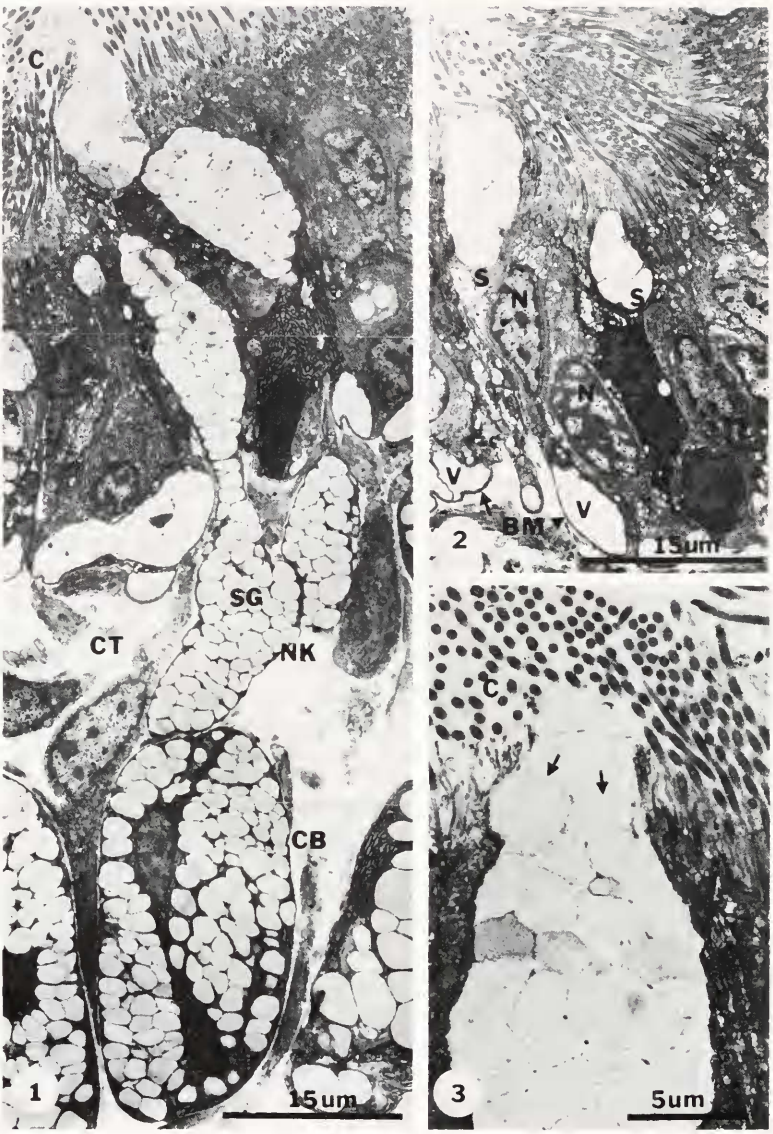


FIGURE 1. The foot epithelium of *Onchidoris muricata* showing the ciliated (C) surface cells and the long-necked secretory cells. These secretory cells have the cell body (CB) in the subepithelial connective tissue (CT). The process (NK) containing secretory granules (SG) extends to the surface of the foot.

FIGURE 2. The cells forming the epithelium have a basal vacuole (V). The nucleus (N) may be centrally or basally located in the cytoplasm. Some of these cells are secretory (S). The entire epithelium is subtended by a basement membrane (BM).

FIGURE 3. Expulsion of mucoid at the foot surface results from fusion of apical granules with plasmalemma and granules below, the separating membranes becoming obliterated (arrow). The mucoid aggregates and is dispersed by cilia (C).

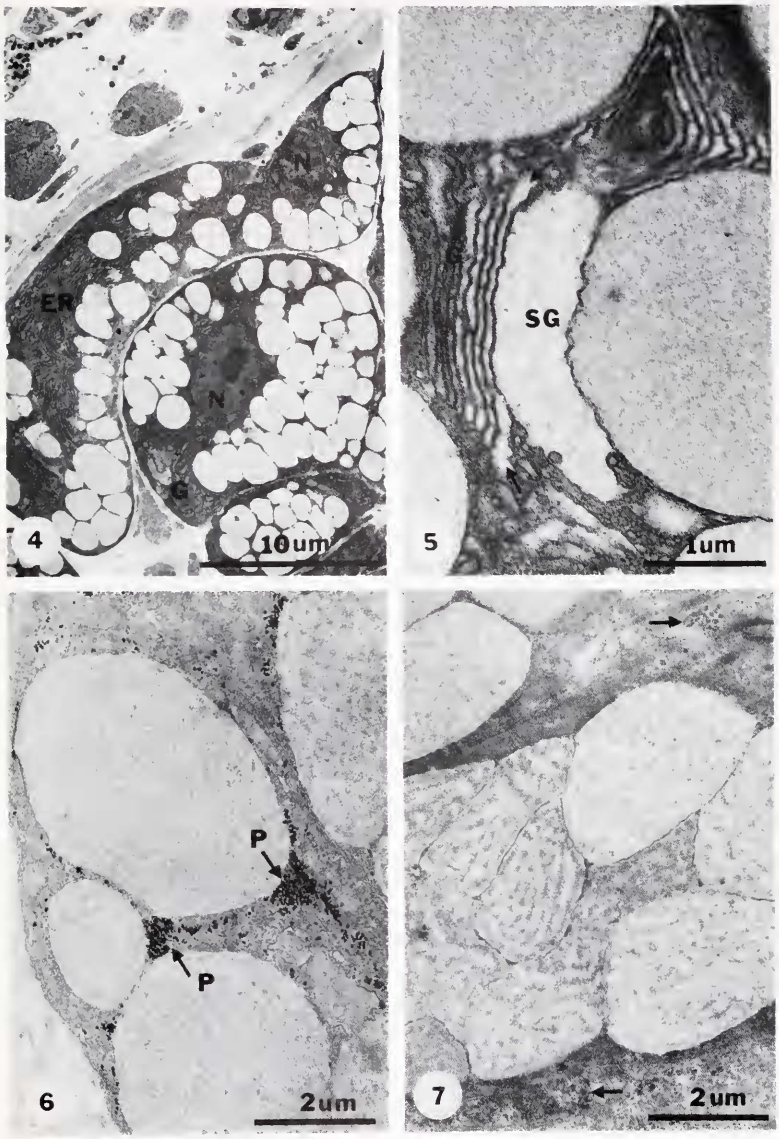


FIGURE 4. A cross section of the long-necked cell body showing the nuclei (N), large amounts of endoplasmic reticulum (ER), Golgi body (G), and secretory granules.

FIGURE 5. The Golgi apparatus (G) of the long-necked cell shows the distending cisternae (arrows) with contents similar to immature secretory granules (SG).

FIGURE 6. The periodic acid-thiosemicarbazide-silver protein (PA-TSC-SP) test is positive for glycogen (P, arrows), but not for the contents of the secretory granules. This test is negative for carbohydrates with obstructing groups.

FIGURE 7. TSC-SP control for the PA-TSC-SP test. Glycogen (arrows) is not positive when periodic acid oxidation is omitted from the test procedure.

*Cytochemistry*

The contents of the long-necked secretory cells were analyzed for periodic acid oxidizable carbohydrate. The ultrathin sections were oxidized with periodic acid and then submitted to thiosemicarbazide for conversion to a derivative that precipitated silver metal from silver proteinate. Observations of cytoplasmic components within the long-necked cells showed that granules of $\alpha$-glycogen were readily oxidized by this procedure and caused significant silver deposition (Fig. 6). The glycogen was often found concentrated around and in between the secretory granules, although smaller amounts were distributed throughout the cytoplasm (Fig. 6). The secretory material in the Golgi apparatus and granules did not show significant reaction in this test (Fig. 6). The fibrillar or granular structure in these organelles was similar to that found in nonoxidized osmium stained tissue (Fig. 5). Controls in which periodic acid oxidation was omitted, but thiosemicarbazide and silver protein included, showed no reaction in the glycogen deposits (Fig. 7). These results demonstrated that this test was specific for periodic acid oxidizable carbohydrate, as demonstrated by the dark staining glycogen. In addition, the PA-TSC-SP tests also showed that the secretory material within the granules did not contain significant amounts of this type of carbohydrate. Presumably, any carbohydrate present had obstructive groups preventing periodic acid oxidation.

To test for possible sulfated polysaccharides that are not sensitive to periodate oxidation, the high iron diamine (HID) test was used on fresh foot tissue. This test is specific for complex carbohydrates esterified with sulfate. The regions occupied by secretory granules in the long-necked cells in osmified tissues were completely stained with HID (Fig. 8). In addition, the mucous blanket over the ciliated epithelium stained similarly (Fig. 8). This test also proved useful in distinguishing the long-necked cells from the shorter epithelial cells. The secretory products in the short cells (Fig. 9) did not stain with HID to the same level of intensity as those in the long-necked cells. Those granules in the short cells which had the greatest stain density appeared fibrillar with darker small cores among the fibrils (Fig. 9). Sometimes several of these cores were present in one granule. Generally all the secretory granules within a specific cell reacted similarly to this test. Thus, each cell type appeared to contain granules all having similar contents rather than mixtures of granules with different products (Figs. 8, 9).

The possible production of glucosaminoglycans by long cells for extracellular use was tested using tannic acid as a preservative for these compounds. The Golgi apparatus was free of any precipitated material, suggesting that these types of compounds were not present in this organelle (Fig. 10). Similarly, the granules arising from the Golgi body also were free of structures suggesting these compounds. Some secretory granules at the apical end of the cell near the plasmalemma were denser than others, but the density appeared to be related to the condensation of the fibrillar contents rather than to specificity of the fixative (Fig. 11). By comparison, the intercellular matrix appeared densely stained, as might result from the presence of intercellular matrix compounds such as hyaluronic acid or other glucosaminoglycans.

The use of alcian blue for staining sulfomucins at the light microscopic level complimented the results obtained with the electron microscope using the PA-TSC-SP and HID reactions. Alcian blue used at pH 2.5 and 1.0 stained the granular product of the long neck cells (Figs. 12, 13). Some of the epidermal cell granules were also stained, but not to the same density or extent as the long-necked cell granules (Fig. 12).

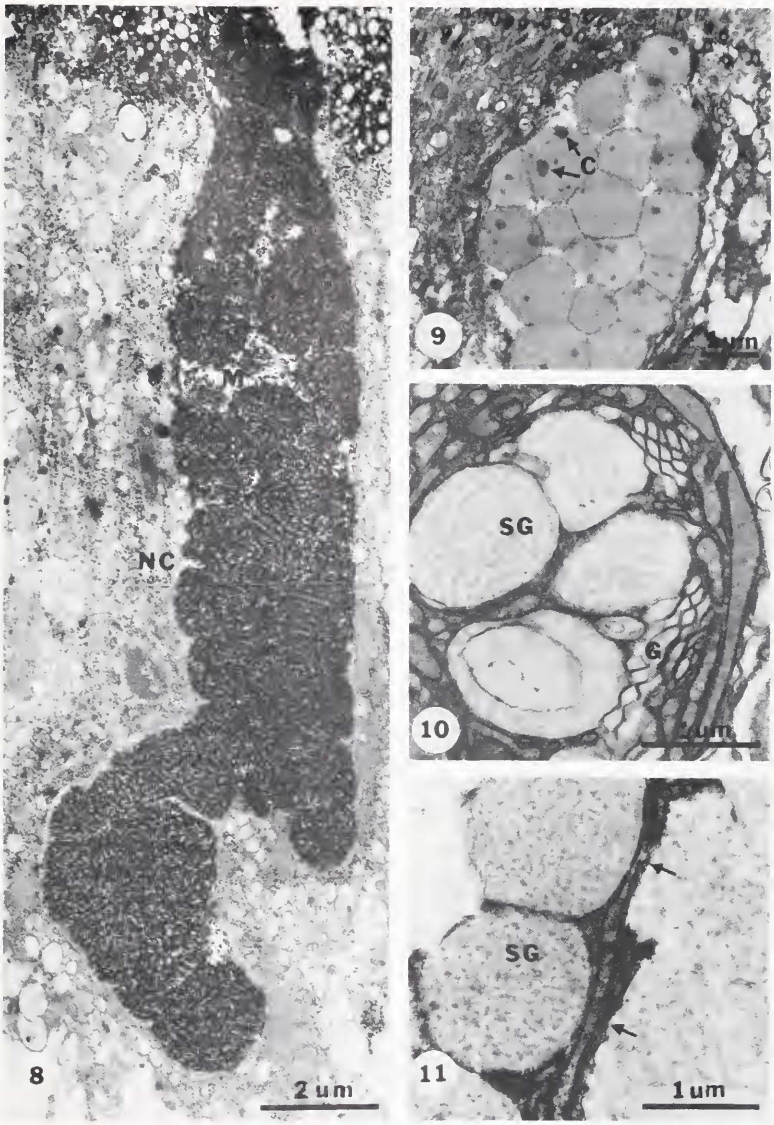


FIGURE 8. The long-necked cells (NC) and mucus (M) showing the intensity of high iron diamine (HID) staining. This stain is for sulfate groups on polysaccharides.

FIGURE 9. Secretory granules in the short epithelial cells show differences in HID staining. Dense cores in the granules (C) are more densely stained than the rest of the matrix.

FIGURE 10. Golgi bodies (G) and secretory granules (SG) do not stain when tissues are fixed with tannic acid. This procedure stains extracellular complex carbohydrates.

FIGURE 11. Tannic acid fixation and staining of intercellular carbohydrates (arrows).

## DISCUSSION

The ultrastructure of mucous cells in marine invertebrates (Storch and Welsch, 1972; Cranfield, 1973a) and secretion in gastropods have been relatively little studied. Biochemically, a wide variety of mucopolysaccharides have been characterized

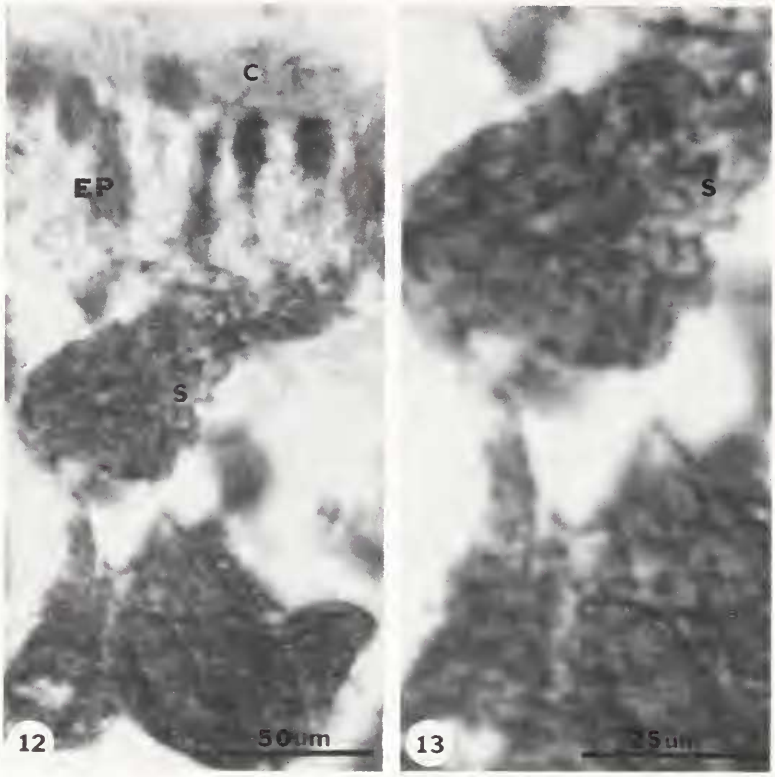


FIGURE 12. Alcian blue 8 GX stained granular product (S) in the long-necked cells. Cilia (c) of the epidermal cells cover the surface of the foot. Epidermal secretory cell (EP) products stain to a lesser extent. Contrast to stain enhanced with contrast interference optics.

FIGURE 13. Detail of several cell bodies of long-necked cells full of secretory product (S). Alcian blue 8 GX, pH 2.5 and 1.0 stain secretory products similarly.

from snails and slugs (Goudsmit, 1972). In most cases, the origin of these materials has not been narrowly defined, and the cells responsible for their secretion have not been identified. The paucity of such studies may have been caused by the confusing variety of secretory cell types distributed over the gastropod body and foot (Porter and Rivera, 1980).

Electron microscopic techniques greatly facilitate studies of individual cell types because the increase in resolution permits close examination of the cellular structures as well as the products of cytochemical reactions which the organelles undergo. The subepidermal long-neck cells in the foot of *Onchidoris* provided a good system for study of secretory cells because they appear isolated as a group and do not have a large variety of different cell types around them. These long-necked secretory cells are found in many nudibranchs (Hyman, 1967). The ultrastructure of these glands has been briefly described in the Mediterranean nudibranch, *Thuridilla hopei* (Storch and Welsch, 1972). However, secretory cells with long processes for directing and expelling secretions are not restricted to gastropods. The acetabular digestive gland cells in the cercarial life stage of schistosomes have a similar structure (Dorsey and Stirewalt, 1971).

The secretory product in the granules of the long-necked cells arises from the Golgi body. While there are considerable cisternae of rough ER in the cell body, no noticeable number of vesicles or granules appear to be closely associated with this organelle. The ER, Golgi body, and secretory granular components resemble those found in secretory cells of other gastropod tissues. Secretory cells in snail multifide gland (Ovtracht, 1967; Ovtracht *et al.*, 1969; Chailley, 1979) and snail hepatopancreas (Rivera, unpublished) have similar profiles. The relatively substantial amounts of rough ER probably contribute considerably to secretory product synthesis (Neutra and Leblond, 1966). In this respect, the long-necked cells resemble goblet cells of mammalian systems (Neutra and Leblond, 1966; Whaley, 1975). Oyster pediveligers also have similar gland cells (Cranfield, 1973a). In all these cases, the Golgi body appears to produce a single type of secretory product or mixture of products which results in similar granule structure throughout the cell. This nudibranch subepidermal foot cell differs from some types of body secretory epidermal cells in which the Golgi apparatus produces two different products (Porter and Rivera, 1980).

The cytochemical tests used to identify the contents in the secretory granules were selected to detect neutral, sulfated, and aminated polysaccharides. Cranfield (1973b) studied the settlement of oyster larvae and found an increase in the acidity of the secretions from the gland cells in the foot of the pedioveliger as it became sedentary. One type of gland cell (DI) in the oyster pediveliger has secretory granules that ultrastructurally resemble the long-neck cell granules (Cranfield, 1973a). The DI cell of the oyster may contribute to adhesion of the larva to the substratum (Cranfield, 1973b). Since *Onchidoris* travels with its foot on the substratum, some adhesion may be required. The presence of strong adhesives like glucosamnoglycans in the secretory granules was ruled out with the tannic acid tests. The use of tannic acid in the fixation procedure has been used successfully for detection of intercellular matrices during animal development (Singley and Solursh, 1980). Tissues of *Onchidoris* treated with tannic acid showed a strong positive reaction in the intercellular spaces. However, the absence of tannic acid reaction products in the Golgi apparatus and the secretory granules exclude the long-necked cells as producers of glucosaminoglycans.

Studies on epidermal mucoid secretory products in other nudibranchs show that the granules are PAS positive at the light microscopic level (Porter and Rivera, 1980). The granules in *Aeolidia papillosa* are particularly susceptible to periodic acid oxidation (Porter and Rivera, 1979). However, the secretory granules in *Onchidoris* do not contain periodic acid oxidizable neutral carbohydrates. This is substantiated by comparing the intensely stained glycogen in the cytoplasm of long-neck cells to the granules that remain unstained after the PA-TSC-SP procedure.

The high iron diamine (HID) test for detection of sulfated carbohydrates was used to indicate sulfate esters commonly found in molluscs (Goudsmit, 1972) and often known components in some types of mucous cells (Spicer *et al.*, 1978). This test has variable staining capacities of individual granules or of different granules within a granule population in a single cell (Spicer *et al.*, 1978). This variability in staining was observed in some of the epidermal secretory cells in the foot of *Onchidoris*. Thus, some variable amounts of sulfation are present in these secretory products. In contrast, the long-necked cells stained homogeneously and intensely. The light microscopic stains with alcian blue substantiate the ultastructural cytochemistry. Since alcian blue stains these secretory granules at both pH 2.5 and 1.0, the products may be interpreted as weakly acidic sulfated mucopolysaccharides (Pearse, 1968).

The sulfated polysaccharide in the long-necked cells of *Onchidoris* may provide lubricity for locomotion and possibly impart some degree of adhesion for attachment to the substrata. The ultrastructural cytochemistry of these cells is similar to skin epidermal secretory cells of the nudibranch *Coryphella rufibranchialis* (Porter, 1980). Adhesion is not a necessary property in this position on the body of *Coryphella*. However, lubricity for reduction of resistance is important. The similarity of the cellular product in these two organisms supports the lubricating function of the product. However, adhesive properties of this product cannot be excluded. The work of Cranfield (1973a; 1973b) on the settlement of oyster larvae indicates a cell type (D-1) with similar structure and chemical properties to the *Onchidoris* subepidermal foot cells. The oyster cell is functional during the active moving stage as well as the cementing stage of the larval life cycle (Cranfield, 1973b). If the product in *Onchidoris* has adhesive properties, they do not appear to be very strong. The ease with which *Onchidoris* may be removed from the substrata and the speed at which they move indicates a greater need of lubricant than adhesive.

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